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Warfarin Inhibition of Vitamin K 2,3-Epoxide Reductase in Rat Liver Microsomes†

Michael J. Fasco, Louise M. Principe, William A. Walsh, and Paul A. Friedman*

ABSTRACT: Warfarin is a potent inhibitor of vitamin K 2,3-epoxide reduction to vitamin K in vitro and in vivo. Dithiothreitol, an in vitro reductant for the vitamin K 2,3-epoxide reductase, antagonizes inhibition of the reductase by warfarin via mechanisms that have not been determined [Zimmermann, A., & Matschiner, J. T. (1974) *Biochem. Pharmacol. 23*, 1033–1040]. Experiments with rat hepatic microsomes were undertaken to characterize the interactions that exist between vitamin K 2,3-epoxide, warfarin, and dithiothreitol. Increasing concentrations of dithiothreitol decreased inhibition of the reductase by warfarin. When dithiothreitol was present prior to exposure of the reductase to warfarin, there was less inhibition than when the same concentration of dithiothreitol was present after its exposure to warfarin. Moreover, maximum inhibition of the reductase by warfarin occurred at a much slower rate when dithiothreitol was present initially. Inhibition of the reductase by warfarin was greater when the substrate concentration was 100 μM vitamin K 2,3-epoxide than when it was 10 μM epoxide. On the basis of these data, we conclude that (i) dithiothreitol reduces either directly or indirectly a critical disulfide within the reductase that is reoxidized during reduction of the epoxide substrate, (ii) warfarin and vitamin K 2,3-epoxide are not competitive with respect to one another, and (iii) warfarin binding, which produces inhibition, occurs solely to the disulfide form of the reductase. Once it is bound, warfarin inhibits further reduction of the critical disulfide by dithiothreitol. Dithiothreitol therefore antagonizes warfarin by maintaining the reductase in the reduced state.

The metabolism of vitamin K to vitamin K hydroquinone and vitamin K 2,3-epoxide has been linked to γ-carboxyglutamic acid formation in the coagulation factors II, VII, IX, and X as well as in proteins of other tissues such as lung, placenta, and kidney (Suttie, 1980). Warfarin and other 4-hydroxycoumarin drugs inhibit γ-carboxyglutamate formation and are used extensively for the prevention of a variety of coagulation-related disorders in humans and in the control of wild rodent populations. Most data suggest that a primary function of the 4-hydroxycoumarin anticoagulants is inhibition of the enzyme vitamin K 2,3-epoxide reductase (Matschiner et al., 1970; Sadowski & Suttie, 1974; Bell, 1978), which, in a sulfhydryl-dependent reaction, reduces the epoxide to vitamin K (Zimmermann & Matschiner, 1974). The vitamin K is then reduced to vitamin K hydroquinone, which functions as cofactor for the γ-carboxylation reaction (Friedman & Shia, 1976; Whitlon et al., 1978; Wallin et al., 1978; Fasco & Principe, 1980).

The epoxide reductase is a membrane protein of the endoplasmic reticulum (Zimmerman & Matschiner, 1974). Although it can be solubilized by a variety of detergents (Siegfried, 1978), purification of the enzyme has not been achieved, and thus, little is known about the molecular mechanisms of either epoxide reduction or inhibition of the enzyme by warfarin and the other 4-hydroxycoumarin anticoagulants. Microsomal reduction of vitamin K 2,3-epoxide requires an exogenous sulfhydryl compound such as dithiothreitol; the reductant for the physiological reaction has not been identified. In addition to its role as reductant, dithiothreitol diminishes the extent of reductase inhibition by warfarin (Zimmermann & Matschiner, 1974). Warfarin binding to the reductase in vitro is extremely strong, however, and is not readily reversed

† From the Center for Laboratories and Research, New York State Department of Health, Albany, New York 12237 (M.J.F. and L.M.P.), and the Department of Pharmacology, Harvard Medical School, and the Charles A. Dana Research Institute and the Harvard-Thorndike Laboratory of Beth Israel Hospital, Department of Medicine, Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts 02215 (W.A.W. and P.A.F.). *Received April 4, 1983.* This research was supported by Grants HL 19772, HL 25066, and HL 27150 from the National Institutes of Health and an award from the Burroughs Wellcome Fund (P.A.F.).

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by a variety of treatments (Lorusso & Suttie, 1972; Fasco & Principe, 1982). Moreover, immediately following the administration of warfarin to rats, hepatic microsomal vitamin K 2,3-epoxide reductase activity is markedly diminished and does not recover fully until many hours after plasma levels of warfarin are no longer detectable (Fasco & Principe, 1982). Rats that developed a resistance to warfarin have been a useful probe of physiologically relevant pathways of vitamin K metabolism and of mechanisms of warfarin–anti-vitamin K activity. A warfarin binding protein exists in normal rat hepatic microsomes that exhibits a greatly reduced affinity for warfarin in microsomes of warfarin-resistant rats (Lorusso & Suttie, 1972; Searcey et al., 1977). Vitamin K 2,3-epoxide metabolism catalyzed by hepatic microsomes of warfarin-resistant rats is also less sensitive to inhibition by warfarin (Friedman & Griep, 1980; Fasco et al., 1982; Hildebrandt & Suttie, 1982). In order to gain insight into the mechanisms of vitamin K 2,3-epoxide reduction and inhibition of the reaction by warfarin, we investigated the effects of dithiothreitol, warfarin, and vitamin K 2,3-epoxide on one another by varying their concentrations and order of addition to hepatic microsomes isolated from rats.

Experimental Procedures

Materials. Racemic warfarin was purchased from Calbiochem-Behring, vitamin K was from Sigma, and emulgen 911 was from Kao Atlas (Tokyo, Japan). Optically pure (*R*)- and (*S*)-warfarin sodium salts and vitamin K 2,3-epoxide were prepared as described previously (Fasco & Kaminsky, 1980). Solutions of vitamin K or vitamin K 2,3-epoxide were 20 mg/mL in aqueous emulgen 911 (10% v/v). Vitamin K hydroquinone was prepared by incubating 0.02 mL of the vitamin K–emulgen 911 solution with 1 mL of 0.2 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 0.15 M KCl, and 0.1 M dithiothreitol buffer, pH 8.5, in a sealed vial under vacuum at 20 °C for 18 h. Analysis of the solution by high-pressure liquid chromatography (HPLC) (see below) demonstrated that at least 95% of the vitamin K initially present was reduced to the hydroquinone. The hydroquinone solutions could be stored at −80 °C for at least 6 months without detectable oxidation or decomposition. The HPLC used was a Waters Associates Model 244 equipped with a recording integrator (Spectra Physics Model 4000) and WISP auto injector (Waters Associates). The column was a radial-pak C_{18} (5-mm internal diameter, 10-μm particle) housed in a radial compression module 100 (Waters Associates). Protein concentrations were determined by the method of Bradford (1976) with commercially available reagents (Bio-Rad).

Assays. The experimental animals were male Wistar rats (250 ± 20 g) and were fed food and water ad libitum and acclimatized to a 12-h on–off light cycle. They were fasted 12 h before being rendered unconscious with nitrogen. The livers were perfused in situ with physiological saline and the microsomes isolated by differential centrifugation as described previously (Fasco & Principe, 1982). The microsomes pelleted by centrifugation at 100000*g* were resuspended in 0.02 M Tris-HCl, 0.15 M KCl, and buffer, pH 7.4, to a concentration of 10 mg of protein/mL and were stored at −80 °C until use.

Vitamin K 2,3-Epoxide Reduction. Reaction mixtures contained 0.6 mL of microsomes and 1.4 mL of 0.2 M Tris-HCl, 0.15 M KCl, and buffer, pH 7.4. Dithiothreitol was added in 0.05–0.5 mL of the same buffer, keeping the volume of the reactions at 2 mL. Reaction was initiated by the addition of vitamin K 2,3-epoxide or dithiothreitol following preincubation of the rest of the components at 25 °C for 1 min. Warfarin, dithiothreitol, and vitamin K 2,3-epoxide concentrations in reaction mixtures were varied as were their order of addition and reaction time at 25 °C. The specific details of each experiment are described in the figure legends. In experiments in which multiple determinations were required, volumes of the reaction mixtures were increased in proportion to the 2-mL reactions, and the preincubation time at 25 °C was increased to 2 min. Reactions were terminated by the addition of 4 mL of 2-propanol–hexane (1:1) to 2 mL of the incubation mixture. The vitamin K 2,3-epoxide substrate and its vitamin K and hydroquinone metabolites were extracted by vortexing the mixture for 20 s. The phases were separated by a brief, low-speed centrifugation, and a 1.5-mL aliquot of the organic phase was evaporated to dryness under a stream of oxygen-free nitrogen. The residue was redissolved in 0.2 mL of 2-propanol and a 0.025-mL aliquot analyzed by HPLC as follows: (solvent A) solvent B–water (1:1); (solvent B) acetonitrile–2-propanol (4:1). The initial condition of 75% B was maintained for 1 min, and the final condition of 100% B was attained linearly over 4 min and was held for an additional 10 min. The column was reequilibrated at the initial condition for 5 min prior to the next injection of sample. The flow rate was 2 mL/min throughout, and detection was at 254 nm. Concentrations of the vitamins were determined by comparisons of integrated areas with those of external standards. The elution times for vitamin K hydroquinone, vitamin K 2,3-epoxide, and vitamin K were approximately at 240, 330, and 550 s, respectively. Total epoxide metabolism was determined from the sum of vitamin K and vitamin K hydroquinone concentrations.

Vitamin K Hydroquinone Epoxidation. Reaction mixtures normally contained 0.6 mL of microsomes and 1.35 mL of 0.2 M Tris-HCl, 0.15 M KCl, and buffer, pH 7.4, containing 20–40 μM (*R*)- or (*S*)-warfarin. Since the hydroquinone substrate solution contained dithiothreitol, the addition of warfarin to reaction mixtures was necessary to prevent the enzymatic reduction of the product, vitamin K 2,3-epoxide, to vitamin K and subsequently back to vitamin K hydroquinone. In the absence of warfarin, little formation of vitamin K 2,3-epoxide was detected. Following incubation at 25 °C for 1 min, reaction was initiated by the addition of 0.05 mL of hydroquinone solution. Variations in this method are described in the figure legends. Products of the reaction were quantitated as described above for epoxide reduction except that detection was at 230 nm since absorption of vitamin K 2,3-epoxide at this wavelength is 3.4-fold greater than that at 254 nm. For experiments requiring the analysis of multiple samples, incubation mixtures were increased in volume in proportion to the 2-mL reactions, and the preincubation time at 25 °C was increased to 2 min.

For reactions run under an atmosphere of nitrogen, flasks containing all the components except that used to initiate the reaction were sealed with a rubber septum and were alternatively evacuated and nitrogen flushed 3 times for 1 min each at 0 °C. The flasks were then preincubated for 2 min at 25 °C before addition of the initiating component through the septum. An atmosphere of oxygen was attained the same way as the nitrogen atmosphere except the final flushing was with oxygen.

Results

With vitamin K 2,3-epoxide as substrate, the rates of metabolite (vitamin K plus vitamin K hydroquinone) formation over 60 min at a dithiothreitol concentration of 25 mM were independent of whether the atmosphere was nitrogen or oxygen (Figure 1). At 5 mM dithiothreitol, the rates of metabolite

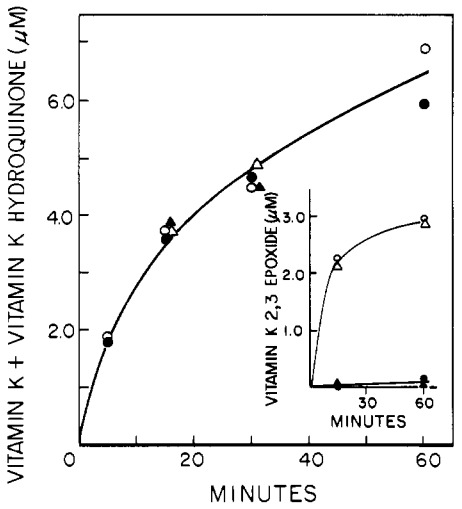


FIGURE 1: Effects of oxygen (○, △) or nitrogen (●, ▲) atmosphere on concentrations of metabolites formed over time from vitamin K 2,3-epoxide and vitamin K hydroquinone (insert). Addition of the substrate (20 μM) initiated each reaction after preincubation of the other components at 25 °C for 2 min. The dithiothreitol concentration was 25 (○, ●) or 5 (△, ▲) mM. (*S*)-Warfarin (20 μM) was included in reaction mixtures containing vitamin K hydroquinone as the substrate. Atmospheres of nitrogen and oxygen were achieved as described under Experimental Procedures. The vitamins were extracted, and their concentrations were determined also as described.

formation after 15 and 30 min were essentially the same as those obtained at 25 mM dithiothreitol (Figure 1). In contrast, with vitamin K hydroquinone as substrate, nitrogen almost completely inhibited its oxidation to vitamin K 2,3-epoxide (Figure 1, insert). Varying the dithiothreitol concentrations (5 or 25 mM) for this reaction also did not affect the rate of epoxide formation over 60 min. Since epoxidation of vitamin K hydroquinone did not proceed under an atmosphere of nitrogen and the rates of epoxide reduction were the same under nitrogen or oxygen, it follows that under the experimental conditions used no hydroquinone formed from vitamin K 2,3-epoxide is cycled back to the substrate. This result is of major importance since much of the data presented below are expressed as percent uninhibited metabolism and the loss of metabolites due to cyclic metabolism would produce erroneous conclusions. It should be noted that Whitlon et al. (1978) have reported that in the presence of dithiothreitol 25–40% more vitamin K was formed from the epoxide in incubations carried out in nitrogen relative to those in air, but the liver microsomes used in those experiments were either from vitamin K deficient or warfarin-treated rats–animals, which, by virtue of the dietary and pharmacological interventions, have elevated epoxidase and carboxylase activities.

Initial experiments examined the effect of warfarin concentration and the order of its addition on the time course of microsomal vitamin K 2,3-epoxide reduction. When the epoxide and 1 or 4 μM (*S*)-warfarin were added to microsomes 1 min prior to the addition of dithiothreitol, near maximal inhibition was attained within 1 additional min, the earliest time point evaluated (Figure 2A). In contrast (Figure 2B), when dithiothreitol was added to microsomes 1 min prior to the simultaneous addition of epoxide and 1 or 4 μM (*S*)-warfarin, inhibition of the reaction increased with time, but even after 16 min, the extent of inhibition was less than that when dithiothreitol was added after (*S*)-warfarin and the epoxide. The pharmacologically more active (*S*)-warfarin enantiomer was no more potent an inhibitor than (*R*)-warfarin when added after the dithiothreitol (Figure 2B) or when added prior to the dithiothreitol (data not shown), which is consistent

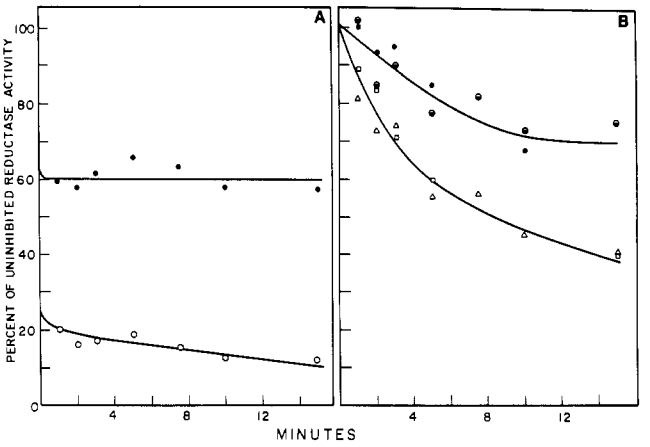


FIGURE 2: Effect of order of addition on warfarin inhibition of vitamin K 2,3-epoxide reductase over time. (A) (●) 1 and (○) 4 μM (*S*)-warfarin were added with the epoxide (20 μM); after preincubation of the reaction mixture at 25 °C for 2 min, 2 mM dithiothreitol was added. (B) (◒, ○) 1 or (△, □) 4 μM (*R*)- or (*S*)-warfarin, respectively, was added at the same time as vitamin K 2,3-epoxide to reaction mixtures that had been preincubated with 2 mM dithiothreitol at 25 °C for 2 min. Reaction mixtures were in a total volume of 16 mL. The vitamins were extracted and their concentrations determined as described under Experimental Procedures.

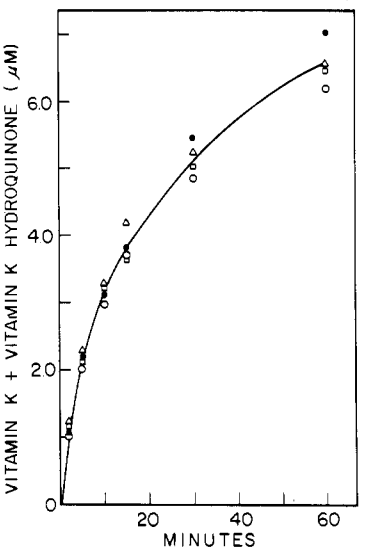


FIGURE 3: Effect of dithiothreitol concentration on sum of vitamin K and vitamin K hydroquinone concentrations formed from vitamin K 2,3-epoxide over 60 min of incubation at 25 °C. Reaction mixtures (16-mL total volume) were prepared and incubated, and the products were extracted and analyzed as described under Experimental Procedures. The epoxide concentration was 20 μM. Dithiothreitol at (●) 1, (○) 5, (△) 10, and (□) 25 mM was used to initiate the reaction after 2 min of preincubation at 25 °C.

with in vivo vs. in vitro results reported previously (Fasco & Principe, 1982).

When dithiothreitol was added to reaction mixtures after 20 μM vitamin K 2,3-epoxide, the rate of epoxide reduction was independent of the dithiothreitol concentration over the range of 1–25 mM (Figure 3). Similar results were obtained when the dithiothreitol was added to reaction mixtures prior to the epoxide except that the rate at 1 mM was slightly below the maximum rate attained at 5 mM and higher dithiothreitol concentrations. Variation in the dithiothreitol concentration did, however, have a pronounced effect on (*S*)-warfarin inhibition of the epoxide reductase. When the dithiothreitol was present in reaction mixtures prior to (*S*)-warfarin and vitamin K 2,3-epoxide, the greater the reductant concentration the less effective was the inhibition by warfarin and the longer the time

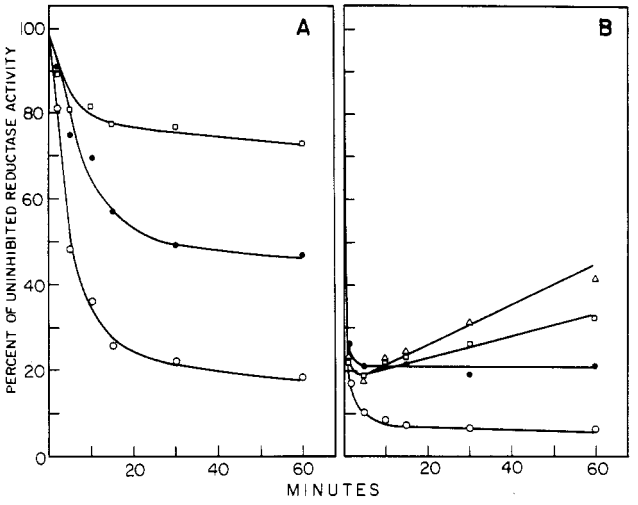


FIGURE 4: Effect of dithiothreitol concentration and order of component addition on (*S*)-warfarin inhibition of vitamin K 2,3-epoxide reductase over time. (A) Dithiothreitol at (○) 1, (●) 5, and (□) 10 mM added before the simultaneous addition of (*S*)-warfarin (4 μM) and epoxide (20 μM) and (B) dithiothreitol at (○) 1, (●) 5, (□) 10, and (△) 25 mM added after the simultaneous addition of (*S*)-warfarin (4 μM) and epoxide (20 μM). Addition of the last component followed preincubation at 25 °C for 2 min. The volume of the reaction mixture was 16 mL. The vitamins were extracted, and their concentrations were determined as described under Experimental Procedures.

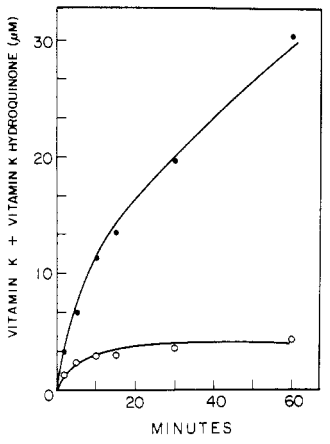


FIGURE 5: Sums of vitamin K and vitamin K hydroquinone concentrations formed from 100 (●) and 10 μM (○) epoxide over time. Reactions were initiated by the addition of substrate following 2-min incubation of the remaining components at 25 °C. The dithiothreitol concentration was 5 mM. The volume of the reaction mixtures was 16 mL. Other conditions were as described under Experimental Procedures.

to attain maximum inhibition (Figure 4A). In contrast, when 4 μM (*S*)-warfarin and vitamin K 2,3-epoxide were added prior to dithiothreitol (1–25 mM), the extent of inhibition after 1 min of reaction was essentially independent of the dithiothreitol concentration (Figure 4B) and was much greater than when the order of addition of dithiothreitol and warfarin–epoxide was reversed (Figure 4A). However, as the time of reaction increased, the extent of inhibition decreased in proportion to the reductant concentration (Figure 4B). At 1–5 min, the level of inhibition remained essentially constant whereas at 10 mM or higher concentrations, the extent of inhibition diminished. These results suggest that dithiothreitol can slowly produce active enzyme from the warfarin–reductase complex.

At 5 mM dithiothreitol, an increase in the epoxide concentration in reaction mixtures from 10 to 100 μM caused a marked increase in the rate of metabolite formation over 60

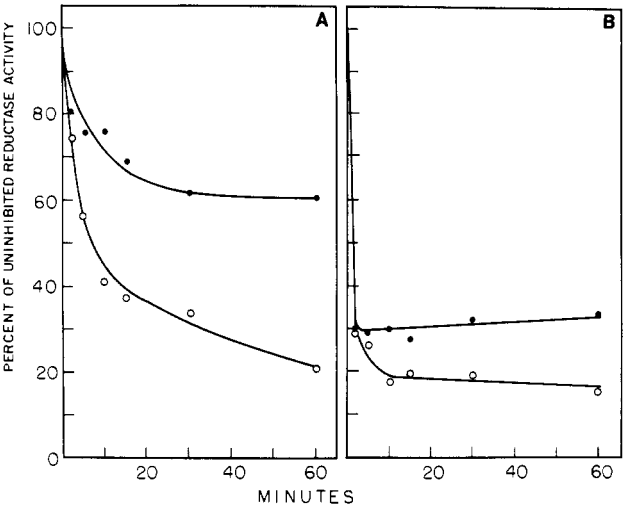
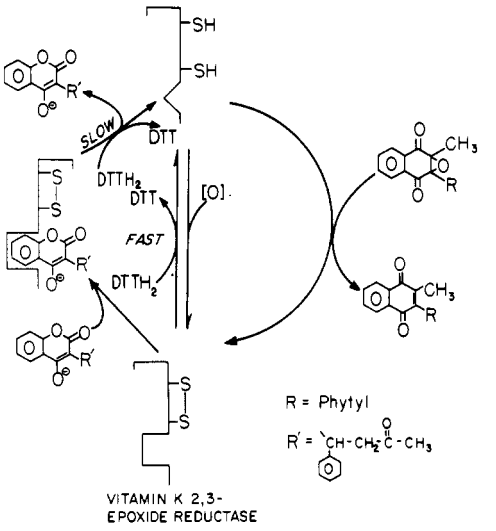


FIGURE 6: Effects of substrate concentration and order of component addition on (*S*)-warfarin inhibition of vitamin K 2,3-epoxide reductase over time. (A) Vitamin K 2,3-epoxide at 10 (●) and 100 mM (○) added before the simultaneous addition of (*S*)-warfarin (4 μM) and dithiothreitol (5 mM) and (B) vitamin K 2,3-epoxide at 10 (●) and 100 μM (○) added after the simultaneous addition of (*S*)-warfarin (4 μM) and dithiothreitol (5 mM). Other conditions were as described under Experimental Procedures.

Scheme I



min of incubation (Figure 5). When 4 μM (*S*)-warfarin was added to reaction mixtures simultaneously with the dithiothreitol and after 10 or 100 μM epoxide, the inhibition was progressive with time and greatest in reaction mixtures containing 100 μM epoxide (Figure 6A). Reversing the order of (*S*)-warfarin–dithiothreitol and epoxide addition produced a level of inhibition at 2 min of reaction that was independent of the substrate concentration (Figure 6B).

Discussion

Numerous studies have demonstrated that warfarin is an inhibitor of vitamin K 2,3-epoxide reductase, but little is known of the molecular basis for the inhibition. A sequence of events leading to vitamin K 2,3-epoxide reduction to vitamin K and warfarin inhibition of vitamin K 2,3-epoxide reductase that is consistent with the results of our investigations is presented in Scheme I. Although vitamin K 2,3-epoxide is depicted here as binding solely to the sulfhydryl form of the enzyme, binding to the disulfide form would also be consistent with the conclusions of these investigations. In the absence of warfarin,

dithiothreitol readily reduces a disulfide bond at the catalytic site of the reductase, either directly or indirectly via another disulfide, and the reduced enzyme catalyzes vitamin K 2,3-epoxide reduction. The plausibility of a mechanism involving sulfhydryl-catalyzed reduction of vitamin K 2,3-epoxide has been recently demonstrated by Silverman (1981a) with model compounds. From his data, the sulfhydryl group produced by reduction of the catalytic site disulfide attacks the epoxide moiety of the vitamin substrate, and the resultant thio–hydroxy intermediate, aided by a neighboring group within the enzyme, rearranges to yield vitamin K and the disulfide form of the reductase.

Inhibition of vitamin K 2,3-epoxide reductase activity by warfarin was greatest and occurred most rapidly when exposure of the enzyme to warfarin occurred prior to its exposure to dithiothreitol (Figures 2 and 4). This strongly suggests that inhibition by warfarin arises solely from its binding to the disulfide form of the reductase. From the reaction sequence depicted in Scheme I, it would follow that conditions that promote formation of the critical disulfide would also promote inhibition of reductase activity by warfarin and conditions that would promote its reduction to the sulfhydryl form would antagonize inhibition by warfarin. Thus, metabolism of vitamin K 2,3-epoxide to vitamin K results in formation of the disulfide form of the reductase, which either undergoes reduction again by dithiothreitol or combines with warfarin, thereby removing it from the pool of active enzyme. The greater reductase inhibition by warfarin at 100 μM epoxide than at 10 μM, particularly following initial exposure of the enzyme to dithiothreitol (Figure 6A), is then due to the more rapid rate of formation of the susceptible disulfide form of the reductase at the higher substrate concentration. In the case in which the reductase was exposed to 1, 5, or 10 mM dithiothreitol prior to warfarin (Figure 4A), both the initial rate of change and the extent of inhibition were affected. Exposure to dithiothreitol following warfarin, however, demonstrated that the extent of inhibition at 1 min of reaction was essentially independent of the reductant concentration over the range of 1–25 mM (Figure 4B). Subsequently, the extent of inhibition changed dependent on the dithiothreitol concentration, and during 30–60 min of incubation, the reductase activity increased at dithiothreitol concentrations of 10 and 25 mM. These results suggest that dithiothreitol affects warfarin inhibition of the reductase by two distinct mechanisms: (i) reduction of a critical disulfide, which precludes warfarin binding and (ii) regeneration of active enzyme from a warfarin–reductase complex. Regeneration of the reductase presumably occurs via dithiothreitol reduction of the same critical disulfide, but in the warfarin–reductase complex the rate of its reduction must be greatly diminished as very high dithiothreitol concentrations are required to achieve even partial restoration of activity.

These data preclude a mechanism whereby warfarin binds covalently to a sulfhydryl group at the catalytic site of vitamin K 2,3-epoxide reductase as has been proposed by Silverman (1981b). As stated previously, warfarin inhibition of vitamin K 2,3-epoxide reductase activity is greatest when the critical disulfide is in the oxidized form, i.e., when it is in a relatively chemically unreactive state. Immediate, covalent bond formation with sulfhydryl groups produced from reaction with endogenous thiol cannot be the cause of this inhibition since preincubation with dithiothreitol, which would analogously increase the sulfhydryl concentration, did not enhance the extent of inhibition at the earliest time investigated (1 min, Figure 2). Instead, exposure of the reductase to dithiothreitol prior to warfarin slowed markedly the rate at which maximal inhibition was attained (Figures 2B and 4A). This antagonism of inhibition cannot result from cleavage of an enzyme–warfarin covalent bond by dithiothreitol because the rate of cleavage would be independent of the order of warfarin–dithiothreitol addition. From the data of Figure 4B, the percent restoration of activity even over 20 min at a dithiothreitol concentration of 25 mM is insufficient to account for the difference in the extent of inhibition produced at 1 or 2 min of reaction when the order of warfarin and dithiothreitol addition are reversed. Further, warfarin inhibition of vitamin K 2,3-epoxide reductase activity is greater at 100 μM epoxide than at 10 μM (Figure 6). If covalent bond formation at the active site were occurring, then inhibition of the reductase at an epoxide substrate concentration of 100 μM should have been less and certainly no greater that inhibition at 10 μM since (i) at the high concentration the epoxide would compete more effectively with warfarin for binding at the catalytic site and (ii) the relatively faster rates of metabolism attained at 100 μM epoxide would produce relatively more of the disulfide form of the enzyme, which would not undergo chemical reaction with warfarin.

Exposure of the reductase to 4 μM warfarin followed by dithiothreitol and either 100 or 10 μM vitamin K 2,3-epoxide produced the same extent of inhibition at 2 min of incubation (Figure 6B). Subsequently, the extent of inhibition increased only in the presence of the higher substrate concentration. These data strongly indicate that warfarin and the epoxide are not competitive with respect to one another for binding at the same site. Warfarin binding must therefore occur at a secondary site that can affect reduction of vitamin K 2,3-epoxide at the catalytic site. A possible consequence of warfarin binding is that a conformational change occurs within the reductase, which dramatically diminishes the rate of dithiothreitol reduction of the critical disulfide bond. Slow reduction of the warfarin–epoxide reductase complex does occur, presumably with liberation of warfarin, since reductase activity can be at least partially regenerated by incubation with high concentrations of dithiothreitol (Figure 4B).

The model proposed in Scheme I does not exclude the possibility that warfarin binding occurs on another protein or enzyme which in some as yet undetermined manner is essential for metabolism of vitamin K 2,3-epoxide. It also does not eliminate the possibility that warfarin forms a covalent bond at a site other than the active site of the reductase although this is unlikely since there is no experimental evidence to support covalent bond formation and warfarin is not a particularly reactive molecule. Only from studies with highly purified vitamin K 2,3-epoxide reductase will it be possible to elucidate completely the mechanisms of vitamin K 2,3-epoxide metabolism and of the allosteric inhibition of the vitamin K 2,3-epoxide reductase by warfarin.

Registry No. Warfarin, 81-81-2; vitamin K 2,3-epoxide, 25486-55-9; dithiothreitol, 3483-12-3; vitamin K 2,3-epoxide reductase, 55963-40-1.

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Spatial Relationships between the Photochemical Reaction Center and the Light-Harvesting Complexes in the Membrane of *Rhodopseudomonas capsulata*†

Jürgen Peters, Jon Takemoto,‡ and Gerhart Drews*

ABSTRACT: The topographical relationships between the photochemical reaction center (RC) and the light-harvesting antenna complexes in the membrane of *Rhodopseudomonas capsulata* were investigated by using reversible chemical cross-linking in conjunction with immunofractionation methods and by using mild detergent fractionation. Cross-linking with the three different reagents 3,3′-dimethyldithiobis(propionimidate) dihydrochloride, dithiobis(succinimidyl propionate), and the photoactivatable compound [(4-azidophenyl)dithio]-succinimidyl propionate yielded essentially identical patterns on two-dimensional polyacrylamide electrophoretograms. In addition to cross-linking between monomer RC subunits H and M and also H and L, close neighbor relationships between H and both polypeptide subunits of the B870 antenna complex as well as the M_r 10000 subunit of the B800-850 complex were implied. A pivotal structural role of H in the coordination of RC-antenna complex interactions is suggested. Mild detergent fractionation was carried out with lithium dodecyl sulfate and Triton X-100. Discrete native pigmented complexes were obtained on polyacrylamide gels containing dodecyl sulfate or Triton X-100, respectively, and also on isoelectric focusing gels containing Triton X-100. The RC was found to be attached to the B870 complex. An RC fragment containing mainly subunit H was found associated with a major part of the B800-850 fraction. In contrast to B800-850, virtually all of the B870 complex was found bound to the RC, probably via both subunits.

The reaction center (RC)[1] and two different light-harvesting antenna complexes are the pigment-protein components of the photosynthetic apparatus of *Rhodopseudomonas capsulata* (*R. capsulata*) (Drews & Oelze, 1981). Light energy is gathered by bacteriochlorophyll *a* (Bchl) and carotenoid bound to polypeptides in the antenna complexes designated B870 and B800-850 according to their near-infrared absorption maxima and transferred to the RC for energy conversion. Biosynthetic investigations and energy-transfer studies with *R. capsulata* and *R. sphaeroides* have led to the idea of the antenna complex B870 being formed at a constant ratio to the RC and being closely associated with it in the membrane (Aagaard & Sistrom, 1972; Monger & Parson, 1977; Feick et al., 1980) whereas the B800-850 complex is synthesized in a variable ratio to the RC depending on growth conditions (Niederman et al., 1976; Schumacher & Drews, 1979; Kaufmann et al., 1982). Energy transfer from B800-850 to the RC is thought to occur via B870 (Monger & Parson, 1977; Feick et al., 1980).

The two pigment-binding polypeptides of the B870 complex of *R. capsulata* have apparent molecular weights of 12000 (12K) and 7000 (7K) (Peters & Drews, 1983a). The B800-850 complex contains three different polypeptides of $M_{r,app}$ 14000 (14K), 10000 (10K), and 8000 (8K), the smaller two of which bind pigment (Feick & Drews, 1979). All four pigment-binding polypeptides of B870 and B800-850 have been isolated and identified by N-terminal sequences (Tadros et al., 1982, 1983; unpublished results) by using the mutant

† From the Institute of Biology II, Microbiology, Albert-Ludwigs-University, 7800 Freiburg, Federal Republic of Germany. *Received March 15, 1983.* The work was supported by grants of the Deutsche Forschungsgemeinschaft (Dr. 29/26-2) and Fonds der chemischen Industrie.

‡ Present address: Department of Biology, Utah State University, UMC 3, Logan, UT 84322. Supported by a fellowship of Alexander von Humboldt Foundation.

[1] Abbreviations: DTSP, dithiobis(succinimidyl propionate); DTBP, dimethyl 3,3′-dithiobis(propionimidate) dihydrochloride; APDP, [(4-azidophenyl)dithio]succinimidyl propionate; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; $LiDodSO_4$, lithium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Bchl, bacteriochlorophyll *a*; RC, reaction center; $M_{r,app}$, apparent relative molecular weight; Me_2SO, dimethyl sulfoxide; TEMED, tetramethylethylenediamine.

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